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Biotransformation of chemicals in water-sediment suspensions – Influencing factors and implications for persistence assessment

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ABSTRACT

Chemicals' half-lives derived from biotransformation simulation studies are central metrics for persistence assessment in international regulatory frameworks. To determine persistence of chemicals released to the aquatic environment, paradigm shifts in recent and on-going revisions of chemical legislations assign increasing importance to OECD 309 simulation studies. OECD 309 studies were designed to target biotransformation in natural water (pelagic test) or in water amended with sediment (suspension test). The suspension test bears several advantages over the pelagic test - most importantly, it employs higher bacterial cell densities, which promote biotransformation of various chemicals at observable rates. Yet, experience with suspension tests is limited. In this study, we followed the fate of 43 pharmaceuticals, pesticides, and industrial chemicals in various suspension test setups and elucidated parameters influencing biotransformation kinetics and half-lives derived thereof. Besides striking intra-study variability between replicates, we found that differences in sediment origin and bacterial cell density resulted in chemical half-lives with up to two orders of magnitude difference, making persistence classification rather uncertain. Yet, data suggested that test systems employing bacterial cell densities at a relatively steady state close to the upper limit of what is commonly observed in natural surface waters yielded increased and more uniform biotransformation of chemicals.

INTRODUCTION

Chemicals can enter surface water bodies through various pathways where they bear the potential to harm aquatic ecosystems. Actual concentration levels of chemicals in surface waters depend on their environmental persistence, defined by a chemical's recalcitrance towards biotic and abiotic transformation [1-3]. In recent years, chemicals' half-lives as indicators of their persistence are becoming increasingly central metrics for hazard and risk assessment in international regulatory frameworks [1, 3-5].

The OECD has developed a series of test guidelines used by professionals involved in chemical persistence assessment and accepted by international regulatory frameworks, e.g., by the REACH regulation of the European Union [1, 6]. Two test guidelines are relevant for chemicals that may enter surface waters: the OECD 308 guideline, which targets biotransformation in aquatic sediments, and the OECD 309 guideline, which assesses biotransformation in the pelagic water body [7, 8].

In November 2018, the European Medicines Agency (EMA) released a draft revision of their environmental risk assessment guidelines for marketing authorization of pharmaceuticals, whose review and revision process is on-going. A major paradigm shift in the new draft guideline is that OECD 309 rather than OECD 308 tests may become the cornerstone of persistence assessment for pharmaceuticals [9, 10]. The draft guideline thus follows REACH regulations, which also emphasize using OECD 309 tests to assess persistence of industrial chemicals [11].

The OECD 309 guideline allows two experimental setups, i.e., testing biotransformation in natural water (pelagic test) or in water amended with sediment to a concentration of up to 1 g solids L^{-1} (suspension test) [8]. To date, OECD 309 studies are mostly carried out as pelagic tests. Pelagic tests pass the validity criteria described in the current OECD 309 guideline when the examined water contains biomass concentrations of minimally 10^3 to 10^4 cells mL^{-1} [8]. However, in fact, bacterial cell densities are higher in most natural surface waters ranging

55 from 10^4 to 10^7 cells mL^{-1}, with a global average of 10^6 cells mL^{-1} [12-15]. Low biomass
56 concentrations in pelagic tests - which are nevertheless deemed to be valid - lead to the effect
57 that very low levels of biotransformation are observed for a large majority of substances and
58 potentially biodegradable compounds may be assessed as persistent. In case a compound is
59 actually biotransformed in a pelagic test - despite the low levels of degrader biomass provided
60 - variability amongst studies with different water samples is high [16, 17]. Previous pelagic
61 tests revealed up to 20-fold differences in half-lives of certain substances, depending on the
62 origin of employed waters [16, 17].
63 Increasing inoculum concentrations by adding sediment-borne biomass may therefore
64 increase the environmental relevance of OECD 309 studies. Furthermore, previous research
65 suggests that increased degrader biomass and pre-exposure of microbial communities to
66 chemicals support their biotransformation, reduce lag phases, and increase reproducibility of
67 laboratory tests [16-23]. However, to the best of our knowledge, so far only a limited number
68 of studies assessed biotransformation of chemicals in water-sediment suspensions and the
69 chemicals were limited to only few model compounds [24-26]. Moreover, these studies did
70 not investigate the influence of bacterial cell densities on biotransformation kinetics, nor did
71 they provide general conclusions on the reproducibility and comparability of suspension tests
72 when conducted in water and sediment sourced from different locations.
73 Therefore, we here explored biotransformation behavior of 43 pharmaceuticals, pesticides,
74 and industrial chemicals that are ubiquitously present in wastewater treatment plant effluents
75 and surface waters [27-31], and hence are environmentally relevant, in various suspension
76 tests, and gathered consistently derived persistence information. To study the effect of testing
77 different microbial communities with varying bacterial cell densities, our experiments were
78 conducted in suspensions sampled from an agriculture- and wastewater effluent-impacted
79 river and a pristine pond at two sediment concentrations, one of them within and one beyond
80 OECD 309 standards. From this, we elucidated experimental parameters that generally

influence biotransformation in laboratory experiments across a wide range of chemicals. We hereby aim to support the on-going EMA revision process with timely experimental evidence on the suspension test of the OECD 309 guideline, which will likely gain significant importance for persistence assessment across relevant regulatory frameworks for various chemicals [9, 11].

MATERIALS AND METHODS

Detailed information on laboratory methods and data analysis are provided in the Supporting Information (SI1, SI2). In brief, suspensions containing 1 and 10 g solids L^{-1} were prepared with water and sediment sampled from the Rhine (R, Mumpf, Switzerland) and from Cressbrook Mill Pond (CMP, Derbyshire, UK). Suspensions are referred to with a code indicating sampling site, sediment concentration in g L^{-1}, and sampling time in case of Rhine suspensions. The lower biomass suspensions are R1-Fall, R1-Spring, and CMP1, the higher biomass suspensions are R10-Fall, and CMP10. Sampling of the Rhine was done twice, i.e., in fall and in spring, to obtain environmental samples with comparable physicochemical properties (Table SI2) but different microbial communities. This investigation of seasonality was done in 1 g solids L^{-1} suspensions to be consistent with current OECD 309 standards.

Sediment was kept in suspension via orbital shaker and additionally via magnetic stirrer in case of CMP sediment (CMP1-Stirrer/ CMP10-Stirrer). Sterile hydrolysis and sorption experiments were conducted alongside the respective biotransformation experiments to distinguish compound removal via biotransformation from abiotic transformation or phase transfer.

Test systems were spiked with a mixture of 43 test compounds to a concentration of 1 µg L^{-1} each. Dissipation of the parent compound was monitored by analyzing up to 11 subsamples taken from the water phase of each experimental vessel over a time course of more than 54

days. Chemical analysis was performed on an Agilent Triple Quad MS coupled to a HPLC system.

Total parent compound residues in experimental systems at a given time were calculated from measured water phase concentrations, considering a sediment-water partitioning coefficient derived from sorption experiments. Compound residues in each experimental replicate as a function of time were fitted to a first-order degradation model considering lag phases to be consistent with data evaluation recommendations in regulatory frameworks [8, 32]. Here, we defined the total system degradation half-life ($DegT_{50,TS}$) as the time interval needed to reach 50% primary degradation, once compound dissipation has started. In contrast, the total system dissipation half-life ($DT_{50,TS}$) was defined as the sum of $DegT_{50,TS}$ and the length of the lag phase.

Bacterial cell densities were determined by sacrificing whole experimental vessels at various time points and enumerating cells, both in the water phase and in the bulk sediment, by using a BD Accuri C6 Flow Cytometer. Due to the necessity of sacrificial sampling, biotransformation experiments were performed with up to 18 replicates and chemical trajectories end at different time points. Still, chemicals' dissipation was monitored in at least two replicates up until the end of each experiment.

RESULTS AND DISCUSSION

Biotransformation in suspension tests. Biotransformation was distinguished from hydrolysis and sorption by comparing biotransformation experiments with their abiotic controls (SI2.2). In agreement with Shrestha et al. [24], results of CMP1/10-Stirrer revealed that keeping sediment in suspension via magnetic stirrer led to grinding of particles and continuously increased sorption of chemicals, which made differentiation between

transformation and phase transfer difficult (SI5). Therefore, data from stirrer experiments was not used further. Based on biotic and abiotic experiments using the orbital shaker instead, dissipation of 35 compounds could be assigned at least partially to biotransformation. Concentration-time series obtained for those compounds are shown in Figure SI1.

Generally, compound dissipation via biotransformation was faster in suspensions with increased sediment content. Least compound losses were observed in CMP1 and R1-Fall - only six substances showed up to 50% removal by the end of those experiments - while dissipation of most substances could be observed in R10-Fall and CMP10. Correspondingly, bacterial cell densities were lowest in CMP1 and R1-Fall with an average of $5x10^6$ and $1.6x10^7$ cells mL^{-1}, respectively. As increasing sediment content led to increased sediment-borne biomass, higher average cell densities of $4x10^7$ and $3.2x10^7$ cells mL^{-1} were measured in R10-Fall and CMP10, respectively. In R1-Spring, we measured an average of $2.2x10^7$ cells L^{-1}, meaning that cell densities did not differ strongly between R1-Fall and R1-Spring. Strikingly though, most compounds dissipated much faster from various replicates of R1-Spring compared to R1-Fall. This suggests that the extent of compound removal via biotransformation is influenced by bacterial cell densities, but also by the composition or activity of the microbial test communities. The latter two parameters have been demonstrated to undergo seasonal variations in surface waters [33-35], which agrees with our findings of varying biotransformation capacities in environmental samples sourced during different seasons.

Besides inter-study variations, we observed drastic differences between replicates of the same study once biotransformation of compounds reached a detectable range. We expressed intra-study variations as the spread between maximum and minimum concentrations of one compound in different replicates at the same time point. Especially during R1-Spring and R10-Fall, intra-study variations increased over time; in R1-Spring, the average spread

between trajectories increased from 270 ng L^{-1} after 13 days to 430 ng L^{-1} after 28 days. Intra-study variations in biotransformation kinetics were lowest in CMP10 with a spread of less than 90 ng L^{-1} regardless of the time point. Described inter- and intra-study variations are exemplarily illustrated for the three compounds atenolol, carbendazim, and diuron in Figure 1.

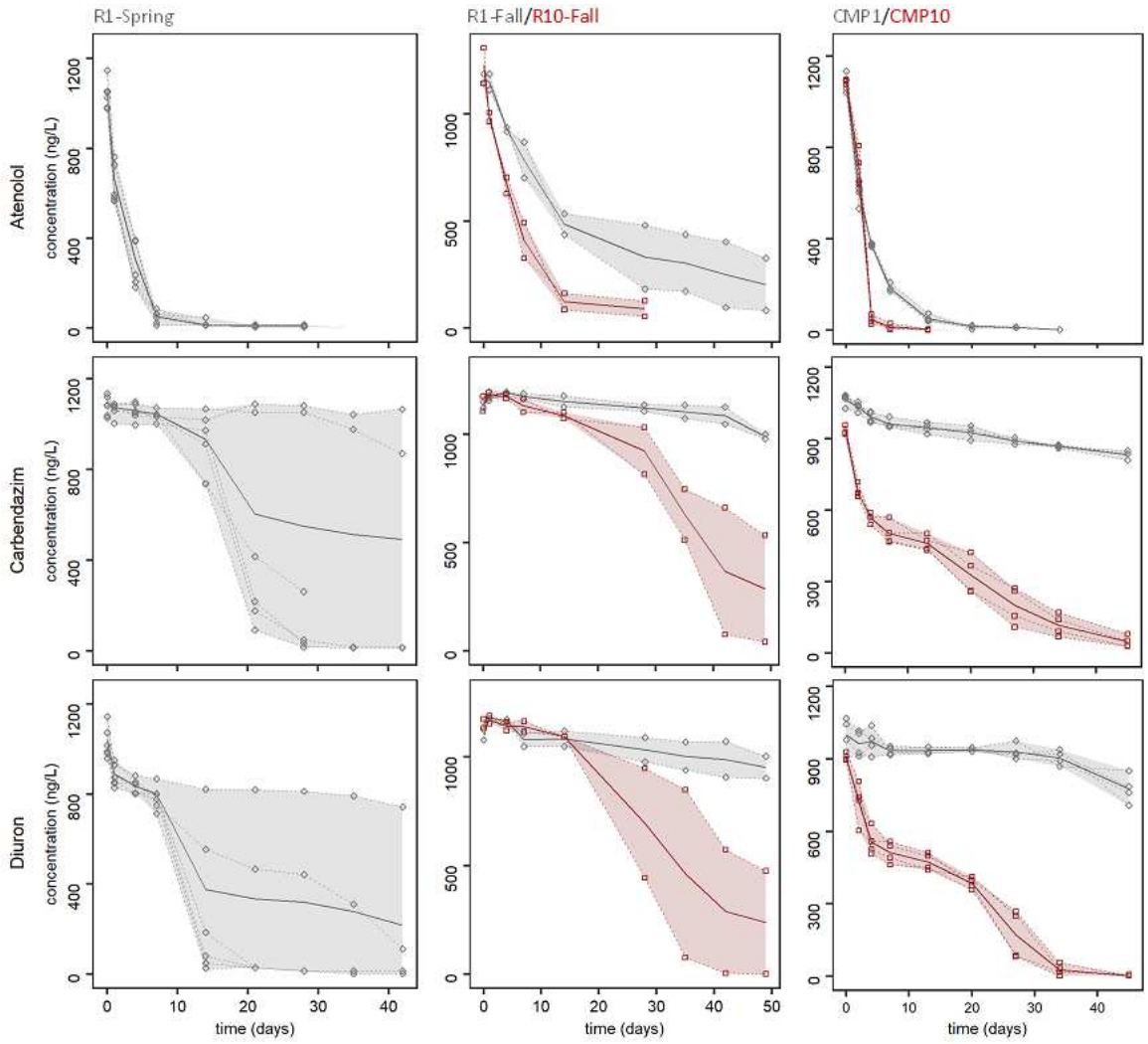


Figure 1. Concentrations of atenolol, carbendazim, and diuron measured in the water phase of R1-Spring, R1-Fall, R10-Fall, CMP1, and CMP10. Suspensions containing 1 g solids L^{-1} are colored in grey with measured data represented as diamonds, and suspensions containing 10 g solids L^{-1} are shown in red with measured data represented as squares. Measurement points belonging to the same experimental replicate are connected with dashed lines. The solid line shows the average concentration calculated from the plotted experimental replicates, shaded areas indicate the spread of the concentrations measured at the same time point.

Interestingly, the extent of variations in biotransformation kinetics coincide with the extent of

169 variations in bacterial cell densities. These variations, expressed as coefficient of variation
170 (CV) calculated from flow cytometry measurements in sacrificed replicates, were greatest in
171 R1-Spring and R10-Fall, with CVs of 74 and 84%, respectively, and lowest in CMP10 with a
172 CV of 44% (SI6, Figure SI4). If we assume that different densities in different replicates at
173 different time points could be considered an approximate indicator of the extent of community
174 dynamics during the experiments, our data suggests that larger community dynamics
175 coincides with increased intra-study variabilities. However, we acknowledge that this
176 hypothesized relationship would certainly profit from actual time series obtained from
177 individual experimental vessels, and additional analysis of not only cell densities but also
178 microbial community composition and activity.

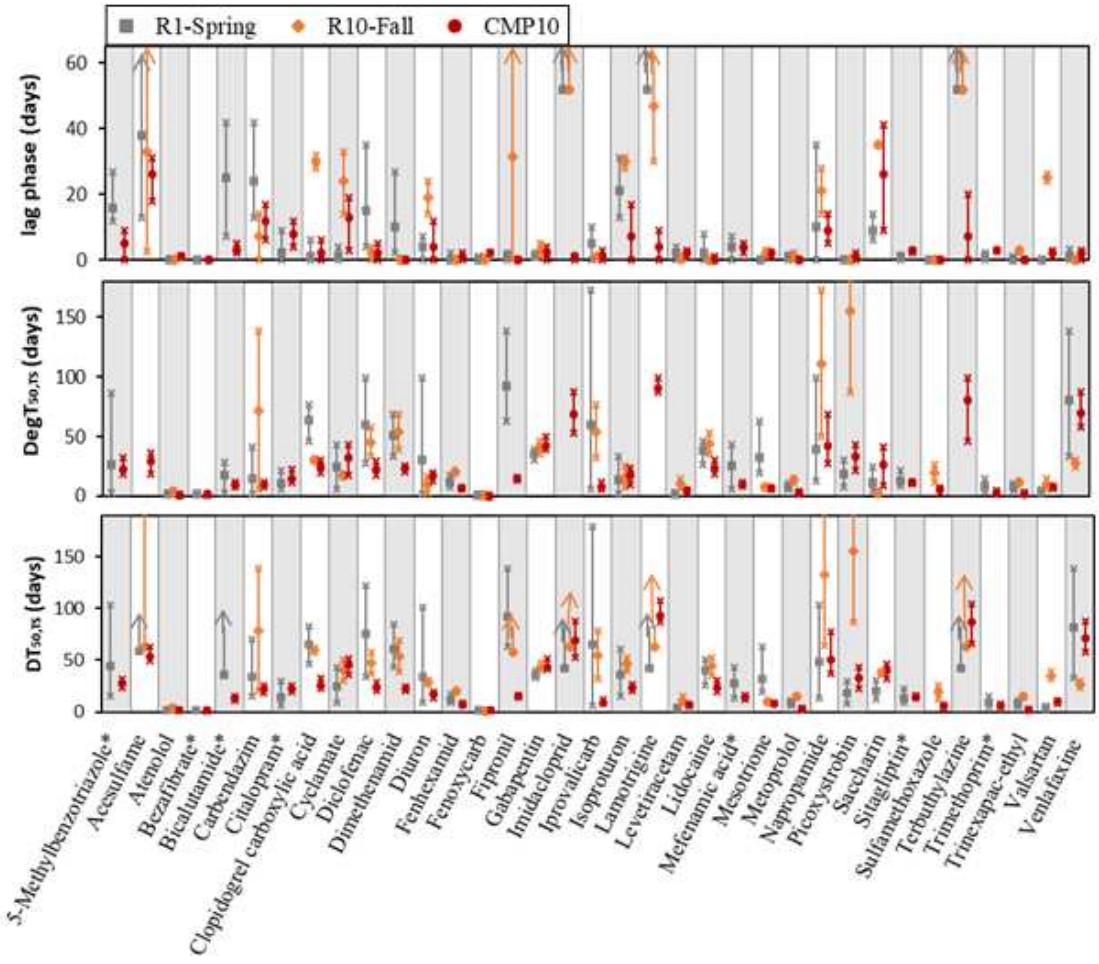
179 Generally, little variation was observed for rapidly degrading compounds, i.e., atenolol,
180 bezafibrate, and fenoxycarb. Literature suggests that those compounds are biotransformed by
181 enzymes widespread among bacteria [36-39], supporting our observation that
182 biotransformation readily occurred in different suspension tests; atenolol was also found to be
183 degradable in OECD 309 pelagic tests [16]. However, inter- and intra-study variations in
184 chemical removal indicate that most of our test compounds seem to have been transformed by
185 enzymes less widespread or only expressed under specific conditions. Rare enzymes have a
186 lower probability of occurrence at lower inoculum concentrations and their emergence in
187 different test systems strongly depends on how the microbial community evolves over time
188 [19, 39-41]. Coherently, a previous study in activated sludge showed that biotransformation of
189 acesulfame, phenylureas (i.e., diuron and isoproturon in our study), and carbendazim strongly
190 depends on the solids retention time and hence community composition, suggesting a need for
191 enzyme activities not always present in activated sludge [36]. In our study, inoculum
192 concentrations above 10^7 cells mL^{-1} were required to increase the likelihood of providing
193 sufficient specific degraders/ enzymes to yield observable dissipation of most compounds
194 from water-sediment suspensions. Qualitatively aligned with our findings, a recently

conducted international ring test showed that increasing cell densities in biodegradation tests with marine waters (OECD 306 [42]) from 10^5 to 10^7 cells mL^{-1} [43] allowed for less variable characterization of biotransformation of five test compounds. With our much broader set of compounds, we showed that elevating inoculum concentrations to the upper limit of what is commonly observed in natural surface waters [15] increased the probability to trigger biotransformation reactions; however, significant inter- and intra-study variations could still occur.

Besides the need for emergence of specific enzymes to remove the majority of our test compounds from aquatic systems, co-metabolic processes might also have promoted compound dissipation during our suspension tests [17, 39, 44]. When comparing suspension tests employing bacterial cell densities sufficient to trigger compound removal via biotransformation (i.e., R1-Spring, R10-Fall, and CMP10), we found shortest lag phases and most uniform dissipation in CMP10. Since average cell densities, at least between R10-Fall and CMP10, did not differ much, we speculate that the comparably elevated carbon and nutrient availability in CMP10 suspensions (Table SI5) might have led to a generally more active microbial community and increased co-metabolism of our test compounds.

Suspension tests in a regulatory context. To further reflect how observed varying biotransformation capacity of microbial test communities influences metrics for regulatory persistence assessment of chemicals derived from biotransformation kinetics in suspension tests (i.e., $DegT_{50,TS}$ and $DT_{50,TS}$), we fitted a kinetic model to trajectories derived from those studies in which we observed significant compound removal, i.e., R1-Spring, R10-Fall, and CMP10 (SI2). A complete listing of all fit parameters and evaluation statistics is provided in SI8, Table SI6. Model fits were generally good with R^2 greater than 0.8 for most evaluated trajectories. Sulfamethoxazole was the only compound for which dissipation clearly did not follow a lagged first-order decay (Figure SI1). Figure 2 presents lag phases, $DegT_{50,TS}$, and

220 $DT_{50,TS}$ for substances biotransformed in R1-Spring, R10-Fall, and CMP10.



221

222 **Figure 2.** Lag phases, $DegT_{50,TS}$, and $DT_{50,TS}$ of biotransformed substances in R1-Spring, R10-
223 Fall, and CMP10. Data for R1-Spring, R10-Fall, and CMP10 is colored in grey, orange, and
224 red, respectively. Squares, diamonds and circles show an average value calculated from the
225 experimental replicates, the error bars extend to the smallest and largest value found for the
226 respective parameter, displayed as crosses. When lag phases exceeded the duration of the
227 experiment, $DegT_{50,TS}$ could not be determined for the respective experimental replicate.
228 When the lag phase exceeded the duration of the experiment in only one replicate, an average
229 $DegT_{50,TS}$ was still calculated with the values from the remaining three replicates of R1-
230 Spring or CMP10. If lag phases exceeded the duration of the experiment in half of the
231 replicates, no averaged $DegT_{50,TS}$ values are shown in this graph. Upwards pointing arrows in
232 lag phase and $DT_{50,TS}$ data indicate that lag phases of at least one replicate exceeded the
233 duration of the experiments and that the actual average as well as the actual maximum are
234 higher than indicated in the graph. Compounds marked with asterisk were not included in the
235 R10-Fall study. Note that the y-axis for the lag phases covers a different scale.

236

237 Lag phases are commonly observed in laboratory biotransformation tests [16, 20, 45]; here,

238 lag phases occurred to various extents for all compounds, except for bezafibrate, and ranged

239 from ~1 day to up to more than 63 days, depending on compound and experiment. Lag phases
240 in the same range were previously observed for several of our test compounds in various
241 OECD biotransformation screening and simulation tests [16, 20, 45]. Here, lag phases were
242 generally shorter in tests carried out with 10 g solids L^{-1}, especially in CMP10. Further, we
243 observed intra-test variations of lag phases, which were most significant in R1-Spring;
244 differences between replicates were greater than 20 days for five compounds (i.e., acesulfame,
245 bicalutamide, carbendazim, dimethenamid, and isoproturon). As discussed previously,
246 biotransformation of at least three of those has been hypothesized to depend on the emergence
247 of specific enzymes.

248 However, current regulatory guidelines do not specify how to consider lag phases when
249 assessing a substance's persistence, i.e., whether $DegT_{50}$ or DT_{50} are to be used as decisive
250 persistence measure [9, 11, 32]. Since lag phases are a sign of microbial adaptation and
251 reduced lag phases could be speculated for compounds continuously or repeatedly released to
252 the aquatic environment (e.g., pharmaceuticals, pesticides, or certain industrial chemicals), it
253 has been argued that $DegT_{50}$ should be used as persistence metric [17, 21-23]. However, our
254 experiments do not directly support this hypothesis as we determined shortest and least
255 variable lag phases in suspensions employing a microbial community sourced from a pristine
256 environment (CMP10). Therefore, and in light of the current lack of understanding of
257 observed variability in lag phases, the use of DT_{50} values to assess persistence would seem the
258 more cautious and environmentally protective approach. It needs to be noted though that
259 $DT_{50,TS}$ of a given compound can range from a few days to over 100 days due to the combined
260 effect of varying lag phases and $DegT_{50,TS}$, see e.g., 5-methylbenzotriazole, carbendazim,
261 diuron, or iprovalicarb in Figure 2/ Table SI6. Similarly, that variations in half-lives of one or
262 two orders of magnitude have previously been observed in OECD 308 or OECD 309 studies
263 for several of our test compounds, including acesulfame, diclofenac, trimethoprim, and
264 venlafaxine, [16, 26, 46].

To evaluate the effect of variable half-lives in a regulatory context, we performed an exemplary persistence assessment according to EMA and REACH criteria [9, 11]. Detailed assessment outcomes are provided in the Supporting Information (SI9). As expected from the significant differences between trajectories observed in various experiments and their replicates, persistence assessment outcomes were ambiguous for about half of the test compounds. Due to differences of up to two orders of magnitude in $DegT_{50,TS}$ and $DT_{50,TS}$ values, persistence assessment outcomes were not only ambiguous relative to the rather strict persistence criteria used in regulatory frameworks of the EU [9, 11], but also relative to higher persistence cut-off criteria used in other legislations, e.g., in the US [1].

Environmental significance. The underlying assumption of persistence assessment based on laboratory simulation studies is that the outcomes reflect compound behavior in the environment [2, 7, 8]. To explore this assumption relative to our results, we compared them to two studies reporting concentration measurements of 12 of our test compounds along the Rhine (i.e., carbamazepine, cyclamate, diclofenac, isoproturon, lamotrigine, saccharin, sitagliptin, sulfamethoxazole, trimethoprim, and three confidential compounds) [27, 47]. In large streams, only compounds with half-lives in the range of the average travel time of all wastewater treatment plant inputs can be expected to show a clear dissipation signature. In case of the river Rhine, where wastewater treatment plants are distributed all along the river, this results in a low average travel time of approximately 7.7 days [47]. As a consequence, only substances with half-lives <10-20 days are significantly removed from the river Rhine. For sulfamethoxazole, sitagliptin, isoproturon, carbamazepine, cyclamate, diclofenac, lamotrigine, and one confidential compound, continuously increasing concentrations along the Rhine have been reported. While persistence classification based on our data was rather uncertain for the first three compounds, the latter five were indeed consistently classified as persistent (Table SI7). Trimethoprim was reported to be removed >80% along the Rhine [47]; accordingly, it was classified as non-persistent in most of our tests. Saccharin stood out in that

291 it showed strong concentration fluctuations along certain parts of the Rhine [27]. This may
292 result from industrial sources with effluent concentrations above typical municipal wastewater
293 concentrations [48], but could also mean that biotransformation of saccharin depends on
294 certain, spatially varying conditions. Indeed, considerable variations in lag phases and
295 $DegT_{50,TS}$ for saccharin indicate that its removal is determined by specific microbiological
296 conditions. The latter would suggest that variable outcomes from different suspension tests
297 may be indicative of strong spatial and temporal fluctuations in biotransformation potential of
298 a set compound in the aquatic environment where composition and activity of microbial
299 communities also vary spatially and temporally [23, 28, 46, 49-51].

300 Still, significant variations make the interpretation of biotransformation study outcomes
301 challenging. It is therefore worth noting that we achieved most robust persistence
302 classifications with a test system containing a 10-fold higher sediment content and at least
303 three orders of magnitude higher bacterial cell densities than minimally required in the current
304 OECD 309 guideline [8]. Our experimental results thus suggest that one approach to yield
305 increased and more uniform biotransformation would be to increase bacterial cell densities
306 and to provide sufficient nutrients in a test system. The fact that increased sediment content
307 increased robustness of suspension test outcomes is also in line with low intra-study variations
308 commonly observed in OECD 308 experiments [24, 46, 52], which employ water-sediment
309 ratios of 3:1 or 4:1 (v/v) [7]. Alternatively, rather than trying to decrease variability in
310 biotransformation tests, different data evaluation tools may allow to interpret test outcomes
311 despite significant variations; in benchmarking, for example, test outcomes for a given
312 compound would be assessed relative to a set of reference compounds with well-established
313 environmental behavior [2]. We hope that further exploration of these emerging concepts will
314 be facilitated with the rich set of biotransformation data that we present here.

315

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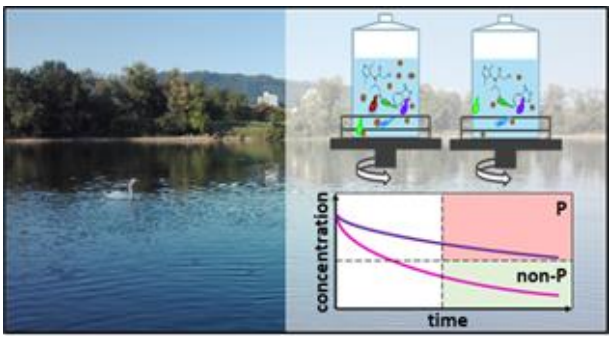

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